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PROFILE

A Feast of Fluorescence

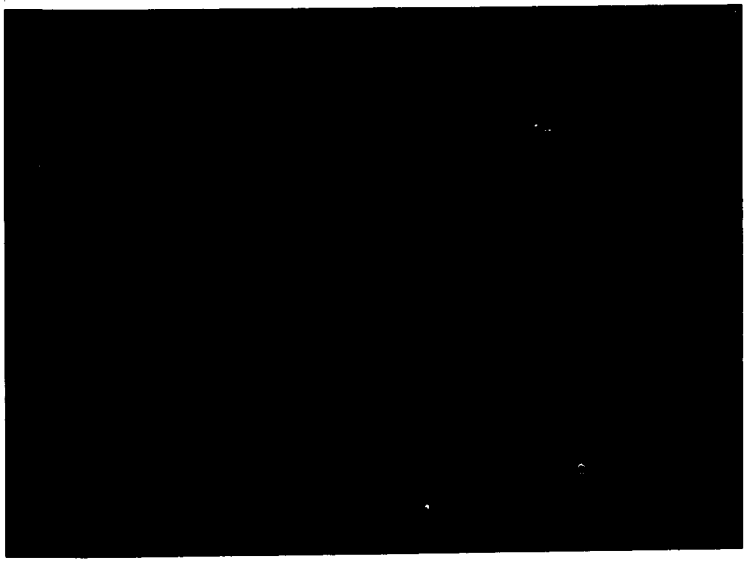
A menu of markers for labeling nucleic acids in four courses

By Deborah Stull

table Labeled Nucleotides

table Complete Labeling Kits

Courtesy of BioCrystal Ltd.



Cancerous human colon tissue (tumor margins) showing non-transformed cells, which are stained with specifically prepared BioPixels.

Not long ago, the options available to scientists for labeling nucleic acids were severely limited. Not only were researchers restricted to working with radioisotopes, but they also had to be satisfied with being able to address only the most basic questions about gene

expression and localization. The development of innovative new technologies and highly specific, nonradioactive labels has changed all of that. In addition to such tried-and-true methods as Northern and Southern blotting, it is possible to label DNA in living cells via in situ hybridization in order to obtain detailed information about the spatial and temporal aspects of gene expression, and to label cDNA with multiple tags for microarray analysis. Countless other applications exist as well. This article offers a sampling of nucleic acid labeling kits, with an emphasis on classical DNA labeling to whet the appetite; reagents used in standard oligonucleotide synthetic chemistry will not be discussed.

Soup or Salad?

In the beginning, there was nick translation.[1] This technique allows researchers to generate radioactive probes in vitro, through the creation of free 3'-hydroxyl ends (or "nicks") within a double-stranded DNA template by DNase I. DNA polymerase I then incorporates labeled deoxyribonucleotides (dNTPs)--in the original case, radiolabeled dNTPs--into the nicks, using the enzyme's 5'-3' polymerase activity. Concurrently, the polymerase's 5'-3' exonuclease activity removes nucleotides in the direction of DNA synthesis, causing the nick to "translate." These combined activities yield a highly labeled DNA probe.

In its early days, nick translation offered researchers a maximum specific activity of 40 percent incorporation. Today, companies such as Promega of Madison, Wis., advertise incorporation rates exceeding 60 percent. Amersham Pharmacia Biotech of Piscataway, N.J., and Roche Molecular Biochemicals of Indianapolis continue to offer nick translation kits but have optimized them for use with either radioisotopes or nonradioisotopic alternatives, to take advantage of the new labels now available. Likewise, Sigma-Aldrich of St. Louis offers its All-in-One™ Nick Translation Labeling Mix, which includes all of the necessary reaction components, including both enzymes and three dNTPs (omitting either dATP or dCTP) in a room-temperature, stable, quick-dissolving lyophilized solid for the production of either radioactive or nonradioactive probes. Other companies have abandoned their nick translation kits, citing a lack of support owing to competition from newer and arguably better methods.

One such method, random priming, was introduced in 1983 by **Andrew P. Feinberg** and **Bert Vogelstein**.[2] In this technique, researchers use a mixture of random oligonucleotides, usually six (hexamers) to ten (decamers) nucleotides long, to randomly prime DNA synthesis along the entire length of the denatured template strand. This method generally results in higher rates of incorporation than does nick translation. The exonuclease-free (Exo-) Klenow fragment of DNA polymerase I is the usual choice for incorporating labeled dNTPs in random priming protocols, though other enzymes are also used. The Ladderman™ Labeling Kit from PanVera Corp. of Madison, Wis., for example, utilizes a thermostable DNA polymerase isolated from *Bacillus caldotenax* YT-G. Random priming can be used to incorporate either radioisotopes or nonradioactive alternatives, and many companies, including GeneCraft of Münster, Germany, Bio-Rad Laboratories of Hercules, Calif., and Invitrogen of Carlsbad, Calif., continue to carry these kits.

Some companies offer clever alternatives to the standard random priming protocol, designed to make the method both easier and faster. For example, Stratagene of La Jolla, Calif., offers the Prime-It® RmT Random Primer Labeling Kit, a version of its Prime-It Random Primer Labeling Kit that can be stored at room temperature for up to six months thanks to a newly discovered thermostable polymerase that is significantly more resistant to thermal degradation than is Klenow polymerase. Similarly, Ready-to-Go DNA Labeling Beads (-dCTP) from Amersham Pharmacia Biotech offer immediate dissolution and are stable at room temperature. This product consists of complete (-dCTP) single-dose reaction spheres predispensed into individual reaction tubes to minimize handling and prevent cross-contamination.

Austin, Texas-based Ambion's Strip-EZ technology is an interesting twist on enzymatic labeling reactions. This system, in which a specially modified Strip-EZ nucleotide is incorporated into the probe along with the labeled nucleotide, has been applied to random priming, in vitro transcription, asymmetric PCR, and reverse transcription labeling techniques. The probes produced in this manner can be stripped from membranes after hybridization using a degradation solution, which cleaves this modified nucleotide. The resulting small probe fragments are then removed from the blot using mild wash conditions. According to Ambion, blots have been stripped and reprobed 10 times or more without loss of target from the blot.

Choose an Entrée

Both nick translation and random priming methods not only traditionally involve the use of radioisotopes, but also depend on the incorporation of modified nucleotides to generate labeled DNA. This reliance on enzymatic activity introduces a number of potentially problematic factors, including the amount and quality of template DNA needed. In addition, polymerases generally prefer unmodified nucleotides to modified ones, thus creating a probe that is only lightly labeled. Finally, neither enzymatic method generates full-length, intact, labeled probes.

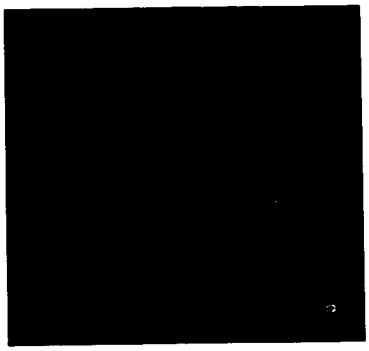
Fortunately, nonisotopic labeling no longer depends entirely on enzymatic reactions. Instead, many companies now offer direct labeling kits for use with a variety of haptens. This technology often involves the use of a labeled linker molecule that intercalates into the template DNA, thereby eliminating the potential pitfalls of enzyme-mediated reactions and providing researchers with one kit that can be used to label multiple types of nucleic acids.

In general, kits vary in the specific molecule used as the linker. For example, the BrightStar™ Psoralen-Biotin Kit from Ambion uses psoralen covalently bound to biotin to crosslink biotin to single-stranded RNA and DNA. The FastTag® System from Vector Laboratories of Burlingame, Calif., employs the disulfide-containing universal linker, FastTag Reagent, photo- or thermally coupled to the template, to attach any thiol-reactive label to the DNA. Vector Laboratories has also recently introduced the 5' End Tag™ labeling system. Unlike the FastTag system, the 5' End Tag kit attaches the label only to the 5' end of the nucleic acid, leaving the 3' end free for extension by polymerase. Conjugation of the label to DNA in this manner avoids potential problems in gel shift assays or in binding to a solid support.

The Flashlight™ Labeling System from Assay Designs Inc. of Ann Arbor, Mich., is another nonradioactive, nonenzymatic labeling system. This system labels nucleic acids, antibodies, proteins, and peptides with chemiluminescent acridinium esters. Once the labeled Flashlight material (Assay Designs offers an extensive collection of options) is bound to the template of choice, researchers can detect the labeled molecules using a "trigger" solution and a simple luminometer.

Several labeling systems use platinum to attach specific labels to DNA. For example, this metal is used in the VersiTag Labeling Systems from PerkinElmer Life Sciences of Boston to label DNA with either dinitrophenol (DNP), a small hapten that yields highly specific probes, or fluorescein. It is also used in the Chem-Link Labeling Kits from Roche Molecular Biochemicals to attach either biotin or digoxigenin.

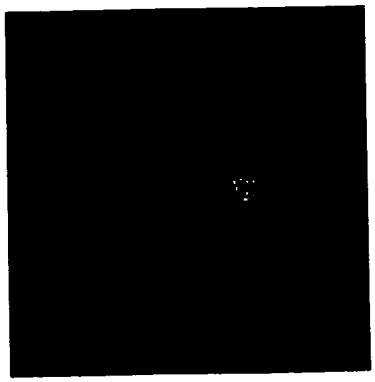
Courtesy of Xiaoquin Chen



α-satellite probes specific to centromeres of chromosomes 1 and 17 labeled by Molecular Probes' ULYSIS Nucleic Acid Labeling Kit.

The technology behind Amsterdam-based Kreatech Diagnostics' ULYSIS® Nucleic Acid Labeling System is based on a modified cis-platin compound, known as the Universal Linkage System (ULS). In cis-platin, four groups are bound to the platinum in a coordination complex. Two of the groups act as leaving groups in a reaction that coordinates the platinum to the N7 of guanine and can thus crosslink DNA. In the ULS compound, a fluorophore or hapten is substituted for one of the leaving groups; as a result, the ULS compounds binds only one guanine base, labeling it with a fluorophore or hapten. A number of labeling kits utilizing this system are available from Kreatech Diagnostics in conjunction with several other vendors. Kreatech carries kits based on biotin, DNP, and fluorescein (in conjunction with Molecular Probes of Eugene, Ore., Roche, and PerkinElmer Life Sciences), Cy3 and Cy5 (in conjunction with Amersham Pharmacia Biotech), rhodamine, and dGreen. This system/linker is designed to bind a vast array of potential marker molecules, thus making it a versatile tool for labeling DNA, RNA, and proteins.

Courtesy of Mindy George-Weinstein



Molecular Probes offers another system that employs both enzymatic labeling and chemical labeling, capitalizing on the advantages of both. The ARES™ DNA Labeling Kits use a two-step procedure in which an amine-modified nucleotide, 5-(3-aminoallyl)-dUTP, is first incorporated into the DNA via traditional enzymatic methods. This amine-modified DNA is then chemically labeled with an amine-reactive fluorescent dye, thus avoiding potential problems with the inefficient incorporation

Stage I quail embryo triple-labeled with Genisphere 3DNA starfish Cy3 probes that contain a recognition sequence for MyoD mRNA (red), the G8 monoclonal antibody and a secondary antibody conjugated with Alexa Fluor 488 (green), and the bis-benzamide nuclear stain (blue).

of bulky labels and resultant lackluster fluorescent labeling intensities. These kits are supplied with Molecular Probes' patented Alexa Fluor® fluorescent dyes.

A Side Dish For the Entrée?

This revolution in nucleic acid labeling is fueled by the wide assortment of new and improved nonisotopic marker molecules, appropriate for both direct (one-step visualization with fluorescence) and indirect (visualization through an interaction with a secondary system) approaches. The first nonradioactive nucleic acid labeling method developed was based on the biotin-avidin/streptavidin complex (biotin-dUTP).[3] Biotin continues to be a favorite labeling molecule, and is featured in kits from companies such as BD Biosciences-CLONTECH Laboratories Inc. of Palo Alto, Calif., and Vector Laboratories.

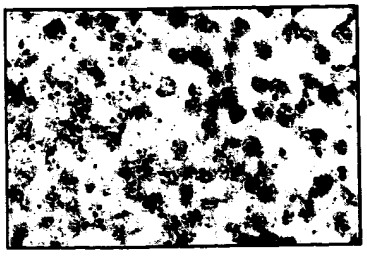
In 1987, Boehringer Mannheim Corp. (now Roche Molecular Biochemicals) introduced a labeling system based on digoxigenin, a steroid hapten isolated from *Digitalis* plants.[4] These plants are the only natural source of digoxigenin; antibodies to digoxigenin, therefore, will not bind to other biological material, resulting in enhanced signal-to-noise ratios. Roche now offers a large collection of DIG-based labeling kits to meet all needs. In addition, Fermentas Inc. of Hanover, Md., and Enzo Diagnostics Inc. of Farmingdale, N.Y., both sell various DIG-based labeling kits. PanVera offers digoxin-based kits in its *Label* IT and HybQUEST *Label* IT product lines. Another indirect labeling option is DNP, available from PerkinElmer Life Sciences, Qbiogene of Carlsbad, Calif., and PanVera.

The first molecule used to label nucleic acids for direct detection was fluorescein, visualized via fluorescence. Fluorescence is superior to radiolabeling in that it is much faster, shows much higher spatial resolution, and makes it possible to visualize several probes simultaneously on the same sample. Fluorescein allows researchers to visualize labeled nucleic acids without an intermediary, thus reducing background noise levels. Initially however, this molecule was not as sensitive as the molecules used in the indirect approaches. Now companies such as PerkinElmer Life Sciences and Molecular Probes sell analogs of fluorescein and other fluorochromes produced by new and improved manufacturing methods. These fluorescent labels now offer sensitivity levels similar to those produced by radioisotopes, but without the high backgrounds originally observed. Horseradish Peroxidase (HRP), available in Rockford, Ill.-based Pierce Chemical Co.'s North2South® Direct HRP Labeling and Detection Kit, provides an alternative direct labeling method.

For the adventurous researcher, there are two more unique labeling options. BioPixels™, from Biocrystal Ltd. of Westerville, Ohio, are novel nanocrystalline fluorescent markers, which can be as small as a few nanometers in diameter. BioPixels

come in multiple colors, each corresponding to a narrow, symmetrical emission spectrum, which can be simultaneously excited by a single ultraviolet light source. They have been developed to bind directly to a number of biological molecules such as proteins, lectins, and nucleotides, and, according to Biocrystal, they also resist photobleaching and produce high-resolution imaging, without background noise.

Courtesy of Nanoprobes

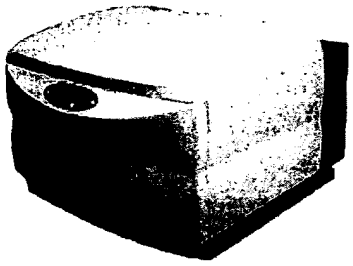


Single copies of HPV 16 in SiHa cells detected by Catalyzed Reporter Deposition™ (NEN Life Sciences) amplification followed by Nanogold-Streptavidin and silver enhancement.

Equally exciting are the Nanogold® Labeling Reagents, including Monoamino Nanogold, Monomaleimido Nanogold, and Mono-sulfo-NHS-Nanogold, available from Nanoprobes Inc. of Yaphank, N.Y. Nanoprobes reports successful labeling of both RNA and oligonucleotides. RNA was labeled through the oxidation of the ribose *cis*-diols (2' and 3' position) to aldehydes with periodate followed by reaction with Monoamino-Nanogold and reduction to form a stable bond, whereas oligonucleotides were labeled through the conjugation of Monomaleimido Nanogold to thiols.[5] Another product, Nanogold-streptavidin, is effective for the detection of biotinylated oligonucleotides via *in situ* hybridization.[6]

The Icing on the Cake

Courtesy of LI-COR



LI-COR's Odyssey™ Infrared Imaging System

LI-COR Inc. of Lincoln, Neb., offers an entirely new detection technology for the researcher who thinks "outside of the box." LI-COR introduced its Odyssey™ Infrared Imaging System at the American Society of Cell Biology meeting late last year for use with its patented infrared technology (IRDyes™) as an alternative to conventional labeling and detection methods. Near infrared (IR) fluorescent dyes (emissions approximately 700 nm and 800 nm) are coupled to a carbodiimide reactive group and attached directly to DNA in a five-minute reaction. The signal is then detected (limit in the low zeptomolar range) by the two detectors of the Odyssey IR Imager. Not only is the signal detection range of this system similar to that observed using chemiluminescence (without the additional steps), but IRDyes offer researchers the unique opportunity to use probes labeled with IRDye700 and IRDye800 together in a two-color Southern blot, something not yet possible with conventional radioactive or chemiluminescent detection techniques.

Today's scientists, therefore, find themselves with a wealth of DNA labeling choices. In addition to the kits mentioned here are numerous kits for more specialized labeling needs, such as for 3'- and 5'-end labeling and cDNA labeling for microarray analysis. These days it seems if a researcher can think up the experiment, there is a kit available

to assist. Happy labeling, and *bon appetit*!

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